PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	
	)	Group Art Unit: 1645
Briggs and Tatum	)	
	)	Examiner: J. Graser
Serial No. 10/055,174	)	
	)	Atty. Docket No. 000295.00014
Filed: January 25, 2002	)	

For: ***LKTA* Deletion Mutant of *P. haemolytica***

DECLARATION OF ROBERT E. BRIGGS UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Robert E. Briggs, declare as follows:

1. I am named as an inventor in the application referenced above. My curriculum vitae is attached as Exhibit 1.

2. For the past 21 years I have studied bacterial respiratory disease of cattle and sheep years. In the past 12 years I have focused on molecular aspects of *P. haemolytica* (now known as *M. haemolytica*), *P. multocida*, and *H. somnus*, which are the principal bacterial etiologic agents involved in respiratory disease of cattle. These efforts resulted in techniques that for the first time made it possible to genetically engineer *M. haemolytica* and *H. somnus*. Using these techniques, we have produced genetically engineered products from each bacterium which have proven useful as attenuated live vaccines and for determination of the role played in

disease by important bacterial virulence factors. Modified-live products have proven to be effective mucosal vaccine candidates against shipping-fever of cattle both in laboratory and field trials. A number of products have been constructed which have demonstrated excellent efficacy in a multivalent vaccine used in sheep and goats, including an intranasally-delivered vaccine for pasteurellosis in bighorn sheep.

3. We conducted four field trials to evaluate oral or intranasal exposure to vaccines containing live lyophilized or previously lyophilized but reconstituted live leukotoxin-deficient *M. haemolytica* bacteria on the health and performance of feedlot cattle freshly placed into an experimental feedlot. In each trial, the leukotoxin-deficient *M. haemolytica* in the vaccine (D153ΔlktA34-378) do not express a biologically active leukotoxin, express a mutant leukotoxin protein that lacks amino acids 34-378, and contain no non-*M. haemolytica* DNA. The trials took place in the period from the Fall of 1998 through the Fall of 2003.

4. The first trial, conducted in the Fall of 1998, tested the efficacy of a vaccine containing reconstituted (formerly lyophilized) live bacteria administered by top-dressing on feed. This trial involved two groups of mixed breed beef calves weighing approximately 500 pounds each. One group (n=100) was procured predominantly from local sales barns by a reputable order-buyer in southwestern Arkansas (high-risk calves). The calves were collected at the order-buyer facility over a period of two days and appeared healthy and alert on the third day when they were processed. The calves were ear-tagged with sequential numbers. To yield an evenly balanced group of vaccinates and non-vaccinates, the odd-numbered calves were cut to a pen separate from even-numbered calves as they left the working chute. The second group (n=120) was collected concurrently from a single ranch in New Mexico, similarly ear-tagged, and cut to separate pens (low-risk calves).

5. The two groups, held at separate facilities about 800 miles apart, were given grass hay (25 pounds) and a pelleted starter ration (150 pounds) spread in 40 feet of feed bunk onto which the *M. haemolytica* vaccine was top-dressed. The vaccine consisted of approximately 50 grams of lyophilized powder containing approximately 10^9 CFU of bacteria/gram reconstituted in 1 liter Earle's Balanced Salt Solution, yielding a dose per calf of approximately 10^9 CFU / head (assuming all the feed is eaten and all the calves eat equally). The liquid was poured onto the feed in the bunks without further mixing into the feed. Non-vaccinated calves were similarly fed without top-dressed vaccine.

6. Vaccinates were held separately from non-vaccinates. Three days later, both groups of calves were trucked to an experimental feedyard in New Mexico (Clayton Livestock Research Center, New Mexico State University). Vaccinates were separated from non-vaccinates on separate decks of a semi-trailer (Arkansas calves) or on separate gooseneck trailers (ranch calves). On arrival at the feedyard, all calves received a clostridial vaccine product, Vitamins A, D, and E, pour-on wormer, and a modified-live IBR/PI-3 (bovine rhinotracheitis / parainfluenza 3) viral product. Nasal swabs and blood serum specimens were collected, and individual calf weights were recorded on the day of arrival and weekly thereafter for a period of 28 days.

7. Upon arrival at the feedyard, two low-risk calves were dead and one low-risk calf was moribund on the semi-trailer from Arkansas. All were non-vaccinates. Over the next three days, five additional non-vaccinated high-risk calves died, bringing the total mortality of this group to 8 calves (16%). Post-mortem showed cranioventral fibrinous pneumonia typical of pneumonic pasteurellosis. Culture results confirmed large numbers of *M. haemolytica*. In some calves, both *M. haemolytica* and *P. multocida* were present. In contrast, only two vaccinated

low-risk calves died (4%) over the course of the trial. One was a calf that exhibited an extended period of diarrhea and weight loss and died 10 days into the trial. This calf had lobular pneumonia, which yielded a pure culture of *P. multocida*. The other calf died near the end of the trial with multifocal pulmonary abscesses (typical of *Mycoplasma bovis*), and culture confirmed *M. bovis* infection. No death loss occurred among the low-risk calves, and their weight gain over the course of the trial exceeded that of non-vaccinates by 25%.

8. We conducted a second trial in the Fall of 2000 in which a lyophilized culture of the leukotoxin-deficient *M. haemolytica* was reconstituted and injected intranasally. This trial tested the effect of intranasal exposure to the live bacteria present in the reconstituted preparation on nasopharyngeal colonization by wild-type *M. haemolytica* in calves at the time of feedyard arrival. The trial involved 200 calves. Half the calves were purchased from an order buyer barn in Arkansas (AR calves; n = 100; mean body weight, 205 kg). The other calves were obtained from a single ranch in New Mexico (NM calves; n = 100; mean body weight, 188 kg). The calves were transported to a feedyard. At the time of arrival, a reconstituted preparation of the leukotoxin-deficient bacteria was administered intranasally to half of each group.

9. Calves were observed daily for respiratory tract disease (RTD). Nasal swab specimens were collected periodically to determine nasopharyngeal colonization status with *M. haemolytica*. Serum samples were assayed for antibodies to *M. haemolytica*. Fifteen AR calves had nasopharyngeal colonization by wild-type MH at the order buyer barn, whereas none of the NM calves had nasopharyngeal colonization. The intranasal exposure to the reconstituted, live leuktoxin-deficient bacteria elicited an increase in serum antibody titers against *M. haemolytica* in NM calves, but titers were less in NM calves treated for RTD.

10. These results demonstrate that exposure of NM calves to the reconstituted, live (previously lyophilized) leukotoxin-deficient bacteria offered protection from nasopharyngeal colonization by wild-type *M. haemolytica*. It is noteworthy that the intranasal vaccination was done after arrival at the feedyard, timing generally considered far too late to offer any benefit by conventional vaccination strategies. This trial is reported in Frank *et al.*, "Effect of intranasal exposure to leukotoxin-deficient Mannheimia haemolytica at the time of arrival at the feedyard on subsequent isolation of M haemolytica from nasal secretions of calves," Am J Vet Res. 2003 May;64(5):580-5 (Exhibit 4).

11. We conducted a third field trial in the fall of 2002 to evaluate a single-dose combination *Mannheimia haemolytica / Pasteurella multocida* edible modified-live vaccine formulation in beef calves. An additional experiment was superimposed within the trial to evaluate a commercial viral combination vaccine given twice before field exposure to calves known to be persistently infected with BVDV. Eighty-four approximately 225 kg mixed English breed beef calves 7-9 months of age were obtained by an order-buyer from a single cow-calf herd in southwestern Arkansas in mid-September. The calves were shipped to the order-buyer the afternoon prior to the day of processing.

12. On the day of processing, all calves were bled by jugular venipuncture for serum and EDTA specimens, wet and dry nasal swab specimens were collected, and rectal temperatures were recorded. Calves received consecutively numbered ear tags. Calves with odd-numbered tags received a modified-live combination viral vaccine product (Jencine 4, Schering-Plough) subcutaneously (left mid-cervical). Consecutive pairs of calves were cut to separate pens as they left the working chute to randomly assemble two groups of 42 calves each, balanced for virus vaccine treatment. These groups were held confined in separate pens overnight after processing,

with water but no feed and with no contact with other calves, to allow the calves to acclimate and increase their desire to eat.

13. The vaccine consisted of *M. haemolytica* strain D153ΔlktA34-378 and *P. multocida* strain P1062ΔhyaE. Late logarithmic growth was obtained in separate 100 ml Columbia broth cultures grown at 37°C with shaking. The cultures were combined and mixed with an equal volume (200 ml) 2X skim milk, which was then frozen at –80°C in a tray for lyophilization. After 4 days of lyophilization at 20°C shelf temperature, the material was crushed into powder and weighed. A portion representing 1 ml of the original culture was reconstituted and assayed by duplicate culture of serial dilutions. Colony counts indicated the 47 grams of recovered lyophilized powder contained 9×10^8 CFU *M. haemolytica* and 8.8×10^8 *P. multocida* per gram. The powder was stored in two ziplock bags, 20 grams each, and kept at –70°C or on dry ice until use. At the time of vaccination, one packet was mixed into 500 ml Earle's Balanced Salt Solution and then top-dressed onto feed in bunks sufficient to allow all calves to feed simultaneously.

14. The next day, about 36 hours after arrival, one group of calves received the oral vaccine preparation top-dressed onto 50 pounds of pelleted calf ration and ½ bale (12 kg) fresh grass hay. The control group of calves received feed similarly without vaccine. Most of the calves fed within 5 minutes of feed placement, and the bulk of the feed was consumed within 20 minutes.

15. Two days after vaccination the two groups of calves were placed into separate grass pastures. No contact was allowed between these two groups, and contact with other cattle was minimized to across-fence contact with few adult cattle. The goal was to minimize the

possibility that non-vaccinates might become exposed to the modified-live vaccine organisms likely to be shed to some degree by the vaccinates and to limit the possibility that wild-type *Mannheimia* or *Pasteurella* might be obtained from non-experimental calves. The calves were held under these conditions for 23 days, then loaded onto a semi-trailer for overnight shipment to the Clayton Livestock Research Center in Clayton, New Mexico. Calves were loaded onto separate decks of the trailer to further minimize the possibility of spreading the vaccine organism to control animals prior to sampling at the feedyard.

16. Eighteen additional head of calves were procured a few days before shipment from local auction markets by the order-buyer. These additional calves were included to supply additional and natural exposure to infectious agents by the principal calves, and were loaded on the fore and aft decks of the trailer to limit their contact with principal calves prior to initial sampling at the feedyard.

17. Upon arrival at the feedyard, the calves were weighed, specimens were collected as previously at the order-buyer facility. Virus-vaccinates were revaccinated, and all calves received wormer (Ivomec, Merial) and blackleg vaccine (Ultrachoice 7, Pfizer). The auction-market calves were ear tagged consecutively as they went through the working chute, as were eight additional calves previously determined to be persistently-infected with bovine viral diarrhea virus. Calves were sorted to six pens based on prior random allocation of ear-tag numbers, which mixed virus vaccine treatment and bacterial vaccine treatment as nearly as possible balanced for both treatment within each pen. Auction-market calves were randomly allocated three per pen to all six pens. Persistently-infected calves were non-randomly allocated two per pen to the first four pens. Pens were physically situated in a single row and were served

by separate feed bunks and waterers, but fences did allow nose to nose contact between adjacent pens.

18. Specimens were collected and body weights were recorded on days 0, 7, 14, and 35 after arrival at the feedyard. Sick animals were identified by pen riders based on a subjective clinical score for nasal and ocular discharge, respiratory rate and effort, body fill, lethargy, gait, and general appearance. Animals deemed clinically ill with undifferentiated respiratory disease were treated with Tilmicosin and penicillin as the primary antibiotics if their rectal temperature was 40 °C or greater. Retreatment was based on the same protocol.

19. At the order-buyer facility, 20 calves were treated for undifferentiated respiratory disease, and two required re-treatment (both non-vaccinates). One control calf died at the order-buyer due to a non-infectious injury sustained to its' cervical vertebrae as it interacted with the working chute during an effort to restrain it to treat undifferentiated respiratory disease. At the feedyard, control calves and bacterial vaccinated calves experienced similar overall pull rates at 32 (n=41) and 27 (n=42) pulls (potentially sick animals) respectively during the 35 day trial. Those which were febrile (and therefore were treated) were 20 and 19 respectively over the course of the trial. Calves which required multiple treatments for respiratory disease (retreatment) numbered 5 for non-vaccinates and 0 for vaccinates over the course of the trial. In comparison, auction-market calves experienced a total of 38 pulls (n=18) of which 22 required treatment, and the persistently infected calves experienced 12 pulls (n=8) of which 6 required treatment. Mortality was limited to two of the persistently infected calves.

20. Serum titers against *M. haemolytica* rose significantly in bacterial vaccinates as compared to control calves (Exhibit 2). Vaccinates' serum titers remained higher than controls, but not auction-market calves, throughout the 35 day trial at the feedyard. Auction-market

calves increased their serum titer, probably in response to natural infection, to closely match vaccinates within one week after arrival. Persistently infected calves did not respond to *M. haemolytica*, possibly because they were immunocompromised.

21. Vaccinated calves exhibited significantly greater weight gain ($p < 0.01$) during the first weeks of feeding compared to control calves and maintained their advantage through the 35 day trial (Exhibit 3). Vaccination improved average gain (kilograms of beef on the hoof gained after day 0) by 5.3 kg at 7 days on feed, 6.5 kg at 14 days, and 6.1 kg at day 35. Both groups of principals showed a gain advantage compared to auction-market calves, about 10.5 kg advantage ($p < 0.01$) among vaccinates and 4.5 kg among non-vaccinates (not significant) than auction-market calves. The principals would be expected to perform better than fresh auction-market calves due to the approximately 28 days of backgrounding which the principals but not the auction-market calves enjoyed.

22. We conducted a fourth trial in the fall of 2003 using a vaccine containing live, dry, lyophilized bacteria. The lyophilized bacterial vaccine was made two years prior to its use in the trial. It was not reconstituted, but was top-dressed dry onto feed. The virus revaccination was carried out at the order-buyer barn two weeks after the initial vaccination. Twelve calves received an experimental killed-virus preparation instead of a modified-live preparation. Otherwise, the methods were nearly identical to those used in the 2002 trial.

23. We are still conducting tests to analyze the results of this trial. Preliminary analysis, however, indicates that vaccination conferred an approximately 5 kilogram weight gain advantage ($p < 0.01$), as was observed in the 2002 trial. Sampling at arrival to the feedyard and after one week at the feedyard indicates that, despite commingling of vaccinates with non-

vaccinates, vaccination dramatically reduced nasal colonization by virulent *M. haemolytica* serotype 1 ($p < 0.001$).

24. Collectively, the four trials described above demonstrate that vaccination with the lyophilized or lyophilized and reconstituted vaccine strain:

- elicits a significant increase in anti-*Mannheimia* antibodies in calves after mucosal administration;
- reduces shedding and carriage of virulent *Mannheimia* in a herd (and therefore reduces the effective infectious load, an important predictor of risk);
- reduces the frequency of re-treatment for undifferentiated respiratory disease (which, according to some experts, is the most relevant indicator of calves which will suffer permanent performance reduction);
- reduces the mortality from pneumonic pasteurellosis; and
- increases the weight-gain of vaccinated calves (a very relevant measure of economic value to cattle producers).

25. I declare that all statements made herein of my own knowledge are true and that I believe all statements made on information and belief are true and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Mar-h 22, 2004
Date

Robert E. Briggs, D.V.M.